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Cyt1Ab1 and Cyt2Ba1 from *Bacillus thuringiensis* subsp. *medellin* and *B. thuringiensis* subsp. *israelensis* Synergize *Bacillus sphaericus* against *Aedes aegypti* and Resistant *Culex quinquefasciatus* (Diptera: Culicidae)

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The interaction of two cytolytic toxins, Cyt1Ab from *Bacillus thuringiensis* subsp. *medellin* and Cyt2Ba from *Bacillus thuringiensis* subsp. *israelensis*, with *Bacillus sphaericus* was evaluated against susceptible and resistant *Culex quinquefasciatus* and the nonsensitive species *Aedes aegypti*. Mixtures of *B. sphaericus* with either cytolytic toxin were synergistic, and *B. sphaericus* resistance in *C. quinquefasciatus* was suppressed from >17,000- to 2-fold with a 3:1 mixture of *B. sphaericus* and Cyt1Ab. This trait may prove useful for combating insecticide resistance and for improving the activity of microbial insecticides.

The pathogenic toxins produced by mosquitocidal strains of *Bacillus thuringiensis* belong to two structurally distinct groups, the crystal (Cry) δ-endotoxins, which are the predominant type, and the cytolytic (Cyt) δ-endotoxins (16). These crystal and cytolytic δ-endotoxins are generally toxic to mosquito larvae, although to various degrees, and are believed to act through the formation of pores in the midgut epithelium by colloid-osmotic lysis (18). The cytolytic toxins are structurally unrelated to the crystal toxins and lyse cells in vitro. Several cytolytic toxins have been identified in mosquitocidal strains of *B. thuringiensis*, including Cyt1Aa and Cyt2Ba1 from *B. thuringiensis* subsp. *israelensis* (15, 33), Cyt2Aa1 from *B. thuringiensis* subsp. *kyushuensis* (19), Cyt1Ab1 from *B. thuringiensis* subsp. *medellin* (31), and Cyt2Bb1 from *B. thuringiensis* subsp. *jegathesan* (7). Of these cytolytic toxins, only Cyt1Aa has been extensively evaluated.

Cyt1Aa is found in the parasporal crystal of *B. thuringiensis* subsp. *israelensis* enveloped with three other mosquitocidal crystal toxins: Cry4A, Cry4B, and Cry11A. Cyt1Aa represents approximately 40% of the protein in the parasporal crystal, whereas each of the three crystal toxins represents about 20% of the remaining protein (17). The toxicity of the individual proteins is lower than the toxicity of the intact parasporal body, and these toxins interact synergistically to produce high mosquitocidal activity (8, 17, 27, 39).

The interaction among the cytolytic and crystal toxins has been implicated as a major factor in the lack of resistance to *B. thuringiensis* subsp. *israelensis* in laboratory and field-selected mosquitoes (3, 4, 11, 12, 14). Experiments revealed that resistance could be readily induced in mosquitoes that were selected with single or multiple crystal toxins but not in mosquitoes selected with the native mixture of crystal and cytolytic toxins in *B. thuringiensis* subsp. *israelensis* (13). Cross-resistance testing of the Cry4- and/or Cry11A-resistant colonies revealed that the selected colonies remained susceptible to *B. thuringiensis* subsp. *israelensis* (35). It was subsequently shown that combinations of Cyt1Aa and Cry4 and/or Cry11A toxins from *B. thuringiensis* subsp. *israelensis* suppressed resistance due to higher levels of synergism against the resistant mosquitoes compared to susceptible mosquitoes (36).

Recently it was shown that sublethal concentrations of Cyt1Aa combined with *Bacillus sphaericus* suppressed *B. sphaericus* resistance (38). *B. sphaericus* is an unrelated, mosquitocidal bacterium that produces a binary toxin with molecular masses of 52 and 43 kDa, which are the binding and toxin domains, respectively (2, 6). This same mixture proved to be highly toxic toward *Aedes aegypti*, a mosquito species that is not normally susceptible to *B. sphaericus* because it lacks a specific receptor for the binary toxin (23). The high activity in both cases resulted from synergism (34, 38).

The interaction of Cyt1Aa with crystal δ-endotoxins and its capacity to suppress resistance to *B. thuringiensis* subsp. *israelensis* and *B. sphaericus* and to extend the host range of *B. sphaericus* raise the possibility that other cytolytic δ-endotoxins may have similar traits. To learn more about the mode of action of cytolytic toxins, we tested Cyt1Ab1 from *B. thuringiensis* subsp. *medellin* and Cyt2Ba1 from *B. thuringiensis* subsp. *israelensis*, alone or in combination with *B. sphaericus,* against *B. sphaericus*-resistant (strain Bs-R) (37) or *B. sphaericus*-susceptible (strain Syn-P) (38) *Culex quinquefasciatus* and against *A. aegypti* (34).

Lyophilized powders of spore-crystal mixtures were used in all tests. Technical powder of *B. sphaericus* (strain 2362) was obtained from Abbott Laboratories (North Chicago, Ill.). Cyt1Ab1 (31) and Cyt2Ba (15) were separately produced in a nontoxic strain of *B. thuringiensis* subsp. *thuringiensis.* Both strains were grown on UG medium (9); the sporulated cells were washed once in 1 M NaCl and twice in distilled water and

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TABLE 1. Toxicities of *B. sphaericus*, Cyt1Ab, Cyt2Ba, and combinations of *B. sphaericus* and Cyt1Ab or Cyt2Ba toward susceptible (strain Syn-P) and *B. sphaericus*-resistant (strain Bs-R) *C. quinquefasciatus*

Toxin(s)	Strain	Exposure time (h)	Lethal concn (μg/ml) (FL)[a]		Resistance ratio[b]		SF	
			50%	95%	50%	95%	LC_{50}	LC_{95}
B. sphaericus	Syn-P	24	0.343 (0.282–0.440)	2.39 (1.46–5.16)				
		48	0.0574 (0.0497–0.0657)	0.252 (0.198–0.347)				
	Bs-R	24	—[c]					
		48	—		~17,421	NC[d]		
Cyt1Ab	Syn-P	24	114.5 (89.2–152)	5,574 (2,922–13,130)				
		48	52.3 (32.1–86.3)	4,739 (1,228–20,689)				
	Bs-R	24	62.8 (41.4–95.9)	1,760 (634–5,270)				
		48	32.9 (22.1–49.3)	1,156 (460–3,114)				
Cyt2Ba	Syn-P	24	31.5 (20.9–47.8)	733 (299–1,863)				
		48	16.3 (10.3–25.7)	238 (99.9–580)				
	Bs-R	24	12.8 (5.1–32.4)	348 (55.5–2214)				
		48	6.77 (3.20–14.3)	89.7 (23.6–344)				
B. sphaericus + Cyt1Ab (3:1)	Syn-P	24	0.126 (0.110–0.143)	0.644 (0.514–0.852)	1.0	1.0	3.6	4.9
		48	0.00326 (0.00238–0.00432)	0.401 (0.239–0.773)	1.0	1.0	23.5	0.8
	Bs-R	24	2.72 (2.30–3.20)	33.9 (25.6–47.6)	21.6	52.6	1,993	10,931
		48	0.132 (0.0707–0.242)	10.5 (2.76–44.6)	40.5	26.2	996	440
B. sphaericus + Cyt1Ab (10:1)	Syn-P	24	0.136 (0.0997–0.185)	0.661 (0.371–1.21)	1.0	1.0	2.8	4.0
		48	0.0175 (0.00837–0.0365)	1.36 (0.227–8.31)	1.0	1.0	3.6	0.2
	Bs-R	24	3.14 (1.94–5.07)	21.3 (8.88–51.7)	23.1	32.2	200	826
		48	0.536 (0.189–1.52)	62.8 (5.20–765)	30.6	46.2	613	184
B. sphaericus + Cyt2Ba (3:1)	Syn-P	24	0.119 (0.0836–0.171)	0.531 (0.258–1.16)	1.0	1.0	3.8	6.0
		48	0.00712 (0.00502–0.0100)	0.289 (0.147–0.604)	1.0	1.0	10.7	1.2
	Bs-R	24	2.47 (2.16–2.83)	15.3 (12.2–20.1)	20.7	28.8	430	2,621
		48	0.0924 (0.0271–0.298)	53.2 (7.13–449)	12.9	184	290	6.7
B. sphaericus + Cyt2Ba (10:1)	Syn-P	24	0.171 (0.116–0.250)	0.943 (0.467–1.93)	1.0	1.0	2.2	2.8
		48	0.259 (0.0156–0.427)	0.675 (0.249–1.85)	1.0	1.0	24.5	0.41
	Bs-R	24	5.03 (2.59–9.76)	151 (31.3–749)	29.4	160	25.5	23
		48	0.357 (0.0846–1.51)	11,217 (51.4–265,158)	1.4	16,617	189	0.08

[a] FL, fiducial limits.
[b] Resistance ratios were calculated relative to the lethal concentrations with the same toxin combination for Syn-P.
[c] —At 1,000 μg/ml, mortality was 0% at 24 h and 17% at 48 h.
[d] NC, not calculated.

centrifuged, and the pellets were lyophilized. Densitometry was used to estimate the amount of toxin protein in the cytolytic toxin preparations from sodium dodecyl sulfate-polyacrylamide gel electrophoresis, using Alpha Ease Version 3.24 (Alpha Innotech Corporation, San Leandro, Calif.) and CA-Cricket Graph III (Computer Associates International, Inc., Islandia, N.Y.). The toxin protein concentration of the Cyt2Ba was estimated to be 0.063 mg/mg of powder. The density of the Cyt1Ab band was on average 1.96-fold greater than that of the Cyt2Ba band (average of three replicates; standard deviation = 0.063 mg), yielding an estimated Cyt1Ab protein concentration of 0.123 mg/mg of powder. For testing, stock suspensions of the powders were prepared in distilled water with glass beads and agitated using a vortex mixer. Stocks were prepared monthly, and 10-fold serial dilutions were prepared weekly. All stocks and dilutions were stored at −20°C when not in use.

Groups of early fourth instars were exposed to a range of concentrations of the spore-crystal powders in 100 ml of distilled water in 237-ml plastic cups. The range usually included 8 to 12 different concentrations that caused mortality of between 2 and 98%, as well as controls. Bioassays were replicated on five different days, with 100 larvae exposed to each concentration. The toxin powder combinations were tested at two proportions, 3:1 and 10:1 (*B. sphaericus* to cytolytic toxin), based on the dry weight of each powder. Mortality was evaluated at 24 and 48 h.

All data were evaluated using a probit program (10, 28). Lethal concentrations with overlapping fiducial limits were not considered to be significantly different. Resistance ratios were calculated by dividing the lethal concentration of the resistant colony (Bs-R) by that of the susceptible colony (Syn-P). Synergism factors (SF) were calculated as described by Tabashnik (30). The SF is the ratio of the expected 50% lethal concentration (LC_{50}) or LC_{95} of the toxin mixture to the observed LC_{50} or LC_{95}, respectively. The expected LC_{50} or LC_{95} was

TABLE 2. Toxicities of *B. sphaericus*, Cyt1Ab, Cyt2Ba, and combinations of *B. sphaericus* and Cyt1Ab or Cyt2Ba toward *A. aegypti*

Toxin(s)	Exposure time (h)	Lethal concn (μg/ml) (FL)[a]		SF	
		50%	95%	LC_{50}	LC_{95}
B. sphaericus	24	106.4 (30.9–343)	434,328 (506–1,853,716)		
	48	7.11 (0.110–290)	34,771 (41–711,138)		
Cyt1Ab	24	59.0 (40.2–87.4)	1,579 (623–4,382)		
	48	32.6 (27.2–39.0)	523 (372–800)		
Cyt2Ba	24	7.71 (6.71–8.71)	27.5 (22.4–36.6)		
	48	6.69 (5.75–7.57)	22.4 (18.4–29.7)		
B. sphaericus + Cyt1Ab (3:1)	24	5.85 (3.55–9.57)	28.6 (11.9–71.1)	15.1	218
	48	3.41 (2.51–4.62)	12.1 (7.34–20.8)	2.6	17.2
B. sphaericus + Cyt1Ab (10:1)	24	1.64 (0.926–2.92)	8.18 (2.88–23.3)	359	1,930
	48	0.990 (0.593–1.65)	4.75 (1.86–12.5)	7.8	969
B. sphaericus + Cyt2Ba (3:1)	24	8.29 (5.17–13.2)	43.4 (18.7–103)	3.0	2.5
	48	1.94 (0.887–4.24)	68.5 (17.2–281)	3.7	1.3
B. sphaericus + Cyt2Ba (10:1)	24	10.9 (7.39–16.0)	136 (61.1–315)	7.1	2.0
	48	3.34 (2.24–4.97)	131 (57.2–324)	2.1	1.7

[a] FL, Fiducial limits

calculated from the weighted harmonic mean of the individual values for the toxins. An SF value of greater than 1 indicated a synergistic interaction between the components in the mixture.

These tests showed that for *C. quinquefasciatus,* powders of Cyt2Ba were more toxic to the susceptible (Syn-P) and resistant (Bs-R) strains than was Cyt1Ab, despite the higher concentration by weight of Cyt1Ab toxin in the powders (Table 1). Although the difference was not statistically significant, both cytolytic toxins were more active toward the Bs-R strain than toward the Syn-P strain.

When *B. sphaericus* was combined with Cyt2Ba or Cyt1Ab at either ratio, the mixtures were highly toxic and generally interacted synergistically (Table 1). SF values ranged from 2.2 to >10,000 after 24 h of exposure and were orders of magnitude higher against the resistant Bs-R (23 to 10,900) than against the susceptible Syn-P (2.2 to 60). SF values were generally lower after 48 h of exposure, and these values ranged from 0.2 to 24.5 and from 0.08 to 996 for Syn-P and Bs-R, respectively.

The cytolytic toxin concentration affected the SF values but not the lethal concentrations (LC_{50} or LC_{95}). For example, lethal concentrations were generally not significantly different when Cyt1Ab was presented at a 3:1 versus a 10:1 ratio. However, SF values, particularly for Bs-R, were affected by the difference in toxin concentration; values were higher at a 3:1 ratio. Lethal concentrations were lower after 48 h than after 24 h of exposure. This effect was more pronounced at the LC_{50}.

The most toxic combination was a 3:1 mixture of *B. sphaericus* and Cyt1Ab. After 48 h of exposure, the LC_{50} against Bs-R was 0.132 μg/ml, and this combination suppressed resistance from >17,000-fold to 2-fold. This represents a 7,500-fold improvement in toxicity toward Bs-R. This same mixture was also more toxic toward Syn-P at the LC_{50} (0.00326 μg/ml) but was not significantly different at the LC_{95}.

Consistent with previous reports, *B. sphaericus* was relatively nontoxic toward *A. aegypti* (20, 22) (Table 2). The Cyt2Ba powder was significantly more toxic than Cyt1Ab against *A. aegypti*, as observed for *C. quinquefasciatus.* When *B. sphaericus* was combined with either cytolytic toxin, toxicity was greatly enhanced. For example, the LC_{95} of *B. sphaericus* was ≈34,000 μg/ml. However, a 10:1 mixture of *B. sphaericus* and Cyt1Ab exhibited an LC_{95} of 4.75 μg/ml, a 7,100-fold increase in toxicity. Combinations of *B. sphaericus* with Cyt1Ab were more toxic than combinations with Cyt2Ba. The enhanced toxicity for all combinations tested against *A. aegypti* resulted from synergism between *B. sphaericus* and the cytolytic toxins. SF values ranged from 2.6 to 1,930 for combinations with Cyt1Ab and from 1.3 to 7.1 for combinations with Cyt2Ba.

The results reported here for Cyt1Ab and Cyt2Ba are consistent with previous work with Cyt1Aa from *B. thuringiensis* subsp. *israelensis.* In those studies, Cyt1Aa suppressed high levels of *B. sphaericus* resistance in *C. quinquefasciatus* (38) and broadened the spectrum of activity to *A. aegypti* when combined with *B. sphaericus* (34). Thus, all three cytolytic toxins from *B. thuringiensis* tested to date have the capacity to synergize *B. sphaericus* against resistant mosquitoes and to enhance toxicity against *A. aegypti.* However, unlike Cyt1Aa, which was not synergistic when combined with *B. sphaericus* and tested against susceptible *C. quinquefasciatus*, both Cyt1Ab and Cyt2Ba interacted synergistically with *B. sphaericus*. This observation is particularly interesting because Cyt1Aa and Cyt1Ab are highly homologous (31). The amino acid differences between Cyt1Aa and Cyt1Ab toxins may provide some important clues to the mechanism of synergism of cytolytic toxins.

Although Cyt2Ba was more toxic per milligram of protein than Cyt1Ab, mixtures of *B. sphaericus* and Cyt1Ab were more acutely toxic and resulted in higher SF values than Cyt2Ba. These high SF values resulted from the low toxicity of Cyt1Ab, as lethal concentrations for the toxin mixtures were generally not significantly different against the same mosquito strain.

Our data differ from those of Thiéry et al. (32), who reported no increase in toxicity against susceptible *Culex pipiens* and *A.*

aegypti for a recombinant strain expressing both *B. sphaericus* 2297 and Cyt1Ab. However, increased toxicity was observed when this recombinant strain was tested against two *B. sphaericus*-resistant colonies, including our Bs-R strain. These differences may be due to the two strains of *B. sphaericus* involved (2362 versus 2297) or to the proportion of Cyt1Ab relative to *B. sphaericus*. Their recombinant strain expressed a 1:2 ratio of *B. sphaericus* to Cyt1Ab (32). Although the precise ratio of toxins in our tests is not known, Cyt1Ab was presented at a lower proportion than the *B. sphaericus* binary toxin.

Recombinant bacterial strains that combine *B. sphaericus* toxins with crystal and/or cytolytic toxins from *B. thuringiensis* have been developed. Some of these strains showed enhanced toxicity against *A. aegypti* and/or against *B. sphaericus*-resistant *C. pipiens* complex (1, 21, 25, 26, 29, 32), while others did not (5, 32). Although the increased toxicity observed with some of these recombinants was attributed to synergistic interactions, no definitive evidence of synergism was provided. The present study and previous work with Cyt1Aa (34, 38) provide substantial evidence that synergism is an important component of the increased toxicity of mixtures of *B. sphaericus* and cytolytic toxins.

The mechanism of this synergism is still not known. Because *A. aegypti* naturally lacks a receptor for *B. sphaericus* (23) and the Bs-R mosquitoes are resistant because their midgut no longer binds the *B. sphaericus* binary toxin (24), cytolytic toxins may promote the binding or insertion of the *B. sphaericus* binary toxins. Further work on the mechanism of toxicity of cytolytic toxins and their interaction with mosquitocidal toxins is needed. Regardless of these unresolved questions, cytolytic toxins may be extremely useful in expanding the host range of *B. sphaericus* and in overcoming resistance to this material in *C. quinquefasciatus.*

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